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The effect of the protein phosphatases inhibitor cantharidin on β-adrenoceptor-mediated vasorelaxation

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1 Cantharidin, an inhibitor of protein phosphatase types 1 (PP1) and 2A (PP2A), increased basal tone of bovine isolated coronary artery rings (CARs) with and without endothelium in a time- and concentration-dependent manner with pEC_{50} values of about 5.1 and 5.2, respectively, for both preparations.

2 β-Adrenoceptor stimulation with isoprenaline (Iso; 0.03–100 μM) or inhibition of phosphodiesterase activity by 3-isobutyl-1-methylxanthine (IBMX; 10–1000 μM), respectively, relaxed CARs precontracted with KCl (75 mM). CARs with and without endothelium showed no difference in the relaxing response to Iso and IBMX, respectively.

3 Cantharidin (3 μM) attenuated vasorelaxation induced by Iso (0.03–100 μM) in CARs with and without endothelium in a time-dependent manner, whereas vasorelaxation induced by IBMX (10–1000 μM) was not attenuated by 3 μM cantharidin.

4 Cantharidin (3 μM) did not affect cyclic AMP content in bovine cultured vascular cells, i.e. coronary artery smooth muscle cells (BCs), aortic endothelial cells (BAECs) and aortic smooth muscle cells (BASMCs), either under basal conditions, after β-adrenoceptor stimulation (Iso) or inhibition of phosphodiesterase activity (IBMX), respectively.

5 Cantharidin inhibited protein phosphatase activity in homogenates from bovine coronary artery rings with a pIC_{50} of about 6.0. In homogenates of bovine cultured vascular cells pIC_{50} values of cantharidin amounted to about 6.5 for BCs, 6.7 for BAECs and 6.7 for BASMCs, respectively.

6 It was concluded that cantharidin differently affects vasorelaxation due to stimulation of β-adrenoceptors (Iso) or inhibition of phosphodiesterase activity (IBMX), respectively. The attenuation of β-adrenoceptor-mediated vasorelaxation by phosphatase inhibition is not due to diminished adenosine 3′:5′-cyclic monophosphate (cyclic AMP) generation but could be evidence for different subcellular compartments of cyclic AMP.

Keywords: Phosphatases; cantharidin; bovine isolated coronary artery rings; endothelial cells; smooth muscle cells

Introduction

Reversible protein phosphorylation is involved in the regulation of a wide variety of cellular processes reflecting the balance between protein kinase and protein phosphatase activities. In the mammalian heart, at least four types of serine/threonine protein phosphatases have been identified: protein phosphatases type 1, 2A, 2B and 2C (for reviews see Shenolikar & Nairn, 1991, and Hunter, 1995). The study of phosphatases has been facilitated by the availability of cell membrane permeable protein phosphatase inhibitors like, for example, okadaic acid, calyculin A or cantharidin. Okadaic acid, a toxin isolated from black sponges (*Halichondria okadai*), causes vasoconstriction in different experimental systems (Shibata *et al*., 1982; Ozaki *et al*., 1987a,b; Gong *et al*., 1992). Cantharidin is another natural toxicant produced by as many as 1500 different species of blister beetles with the spanish fly *Cantharis vesicatoria* probably being the best known source (Honkanen, 1993). Moreover, cantharidin is a potent vesicant and toxicant in mammals with structural similarity to commercial herbicides like endothall and endothall thioanhydrid. The inhibition of protein phosphatases *in vivo* may be the cause of sublethal effects and death (Li & Casida, 1992; Polettini *et al*., 1992). Recently, it has been demonstrated, that cantharidin increases force of contraction in guinea-pig isolated papillary muscles (Neumann *et al*., 1995) like okadaic acid (Neumann *et al*., 1993) and calyculin A (Neumann *et al*., 1994). Moreover, cantharidin inhibits type 1 and 2A phosphatase activity of phosphatases purified from guinea-pig ventricles and increases the phosphorylation state of various target proteins, e.g. phospholamban (Neumann *et al*., 1995). In contrast to okadaic acid, the effects of cantharidin on contractility, second messenger accumulation and protein phosphatase activity in vasculature have hitherto not been investigated. Thus, the effects of cantharidin on contractility of bovine isolated coronary artery rings and activity of phosphatases from bovine coronary arteries and cultured vascular cells were studied. Based on these data we investigated the interaction between β-adrenoceptor-mediated vasorelaxation and phosphatase inhibition in bovine isolated coronary artery rings (CARs) with and without endothelium.

Methods

Preparation of bovine isolated coronary artery rings (CARs) and experimental protocol

Bovine hearts were obtained from a local slaughterhouse. Immediately after removal from the animals, hearts were placed into Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 118, $NaHCO_3$ 25, $CaCl_2$ 2.5, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 11.1 and ethylenedinitrilotetraacetic acid 0.026. Left coronary arteries (anterior descending branch) were cleaned of connective tissue and cut into rings with an approximate internal diameter of 4 mm (approximately 70 mg wet weight). The endothelium was removed by rubbing the intimal surface with the tip of a forceps. Rings

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were mounted in organ chambers similar to those used by Mügge & Harrison (1991) and changes in isometric force of contraction were recorded. CARs were allowed to equilibrate in KHS for 60 min under a basal (resting) tension of 20 mN (Obi *et al*., 1993) which allowed optimal contractions to KCl (75 mM) to be produced. After washout the presence of an undamaged endothelium was checked by relaxation to bradykinin (1 μM) after precontraction with prostaglandine $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μM). Rings showing less than 50% relaxation to bradykinin were discarded as having partially damaged endothelium. Supposedly endothelium-free rings showing more than 10% relaxing response to bradykinin were excluded from further experiments. CARs were then exposed to cantharidin or dimethylsulphoxide (DMSO, solvent control) for one hour and contracted once again with KCl (75 mM) without correction of NaCl in the KHS. Then isoprenaline (Iso) or 3-isobutyl-1-methyl xanthine (IBMX), respectively, were added and changes in force of contraction were recorded. All experiments were performed in the presence of indomethacin (10 μM) and atropine (10 μM) (Greenberg *et al*., 1987; Obi *et al*., 1993; Auch-Schwelk *et al*., 1993) to prevent generation of vasoactive prostaglandins and to inhibit cholinergic effects, respectively. The vasoconstriction of CARs due to the high potassium was most probably not mediated by the release of noradrenaline from sympathetic nerve terminals, because noradrenaline (1–100 μM) only caused relaxation not contraction in bovine isolated coronary artery rings (data not shown).

Culture of bovine aortic endothelial cells

Bovine aortic endothelial cells (BAECs) were obtained by collagenase digestion as described by Makarski (1981), by use of Worthington CLS 2 collagenase (0.1%). Thoracic aortae from freshly slaughtered cows were obtained from a local slaughterhouse. Immediately after removal from the animal, aortae were flushed with ice-cold sterile phosphate buffered saline solution without Ca^{2+}- and Mg^{2+} salts (PBS; pH 7.45) containing penicillin (100 iu ml^{-1}) and streptomycin (100 μg ml^{-1}). Aortae were cleaned of fat and connective tissue, longitudinally dissected and fixed on a polystyrene plate wrapped with aluminium foil. Segments were rinsed with ethanol (70%) to prevent contamination and to eliminate blood cells. Endothelial cells were carefully scraped off with a scalpel, suspended in endothelial cell culture medium (ECM) composed of medium 199 containing foetal calf serum (20%), L-glutamine (100 μg ml^{-1}), amphotericin B (4.3 μg ml^{-1}), penicillin (100 iu ml^{-1}), streptomycin (100 μg ml^{-1}) and gentamicin (50 μg ml^{-1}) and centrifuged for 10 min ($366 \times g$; 4°C). The supernatant was discarded and the pellet was treated with 20 ml collagenase (0.1%) for 10 min at 37°C. The resulting collagenase/cell suspension was immediately mixed with an equal volume of ECM and centrifuged again at 4°C to stop further digestion. Cells were seeded in tissue culture flasks and grown in a humidified incubator under an atmosphere of 7.5% CO_2/21% O_2 at 37°C. Culture medium was exchanged the next day and subsequently replaced every second day. Cells reached confluence within 5 to 7 days forming monolayers with cobblestone appearance. Because direct identification of endothelial cells by light microscopy can be misleading, cells were characterized by staining with an antibody to factor VIII-related antigen (data not shown). Cells were used for the experiments without any passage or after the first passage.

Culture of bovine vascular smooth muscle cells

Smooth muscle cells from bovine aortae and coronary arteries were obtained by using an explant method (Salih *et al*., 1992; Ebersole *et al*., 1993). After removal of the endothelium with a scalpel, pieces of the arterial wall, about 2–4 mm^2, were transferred into tissue culture flasks and allowed to dry for 15–30 min at 37°C to improve adhesion to the surface. Then smooth muscle culture medium, composed of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum, L-glutamine (584 μg ml^{-1}) and antibiotics as described for endothelial cells, was carefully added. Cell growth could be detected after 7 to 10 days. Cells were subcultured with trypsin (0.05%)/EDTA (0.02%)-solution. For experiments only cells between passages 2 and 6 were used. Identity and purity of cultured cells was confirmed by light microscopy and immunohistochemistry by use of a monoclonal antibody directed against α-smooth muscle actin. Immunohistochemical findings were confirmed by Western blotting with the same antibody showing a single band at approximately 40 kDa (Somlyo & Somlyo, 1992) (data not shown).

Determination of cyclic AMP content

Subconfluent monolayers of bovine vascular cells were incubated for 60 min in culture medium containing cantharidin or DMSO (solvent control). Cells were harvested, suspended in PBS and transferred into microcentrifuge tubes. Stimulation of vascular cells (30 min, 37°C) was performed by using either isoprenaline or 3-isobutyl-1-methylxanthine, both dissolved in ascorbic acid (0.1%), in the presence of cantharidin (3 μM) or DMSO (control cells). Reaction was stopped by addition of 0.1 M HCl and heating at 95°C for 10 min. Tubes were cooled on ice for 10 min, centrifuged at 14,000 *g* for 15 min and aliquots of the supernatants were used for determination of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) content. The resulting pellets were dissolved in 0.1 N NaOH and protein contents were determined according to Bradford (1976). Determination of cyclic AMP content was performed as described previously (Neumann *et al*., 1989). All data were referred to protein content.

Determination of protein phosphatase activity

Preparation of homogenates Freshly prepared bovine coronary arteries were powdered in liquid nitrogen and homogenized in medium containing ethylenediaminetetraacetic acid 4 mM and β-mercaptoethanol 0.1% three times for 30 s each with a Polytron PT-MR 3000 (Kinematica AG, Littau, Switzerland). Samples were centrifuged for 20 min at $14{,}000 \times g$ (4°C). Bovine vascular cells were trypsinized, collected by centrifugation at $366 \times g$ for 10 min (4°C) and homogenized in buffer containing (in mM): Tris-HCl (pH 7.4) 20, ethylene glycol-bis(β-aminoethylether)-N,N,N,N-tetraacetic acid 2, ethylenedinitrilotetraacetic acid 5, benzamidine 1, phenylmethylsulphonylfluoride 0.5 (both used as protease inhibitors) and β-mercaptoethanol (0.1%) by pasting the cells through a needle (0.4×19 mm). Samples were centrifuged at $12{,}000 \times g$ for 30 min. The supernatants are called homogenate throughout this work.

Protein phosphatase assay Protein phosphatase activity was determined as described previously (Neumann *et al*., 1993). In brief, phosphatase activity was measured with [^{32}P]-phosphorylase a as substrate. The incubation mixture contained (in mM): Tris-HCl (pH 7.0) 20.0, caffeine 5.0, ethylenediaminetetraacetic acid 0.1 and β-mercaptoethanol 0.1% (vol/vol). The reaction was started by adding homogenates of bovine coronary arteries or homogenates of bovine cultured vascular cells, respectively. Reaction was stopped by addition of 50% trichloroacetic acid. Precipitated protein was sedimented by centrifugation. The supernatant was counted in a liquid scintillation counter.

Experiments on cyclic AMP accumulation and protein phosphatase activity could not be performed in bovine cultured artery endothelial cells because of the low yield of these cells and the large quantities of these cells needed for the experiments.

Chemicals

Collagenase Typ CLS 2 was obtained from Worthington (Freehold, U.S.A.). Medium 199, DMEM, amphotericin B,

atropine, benzamidine, cantharidin, indomethacin, N^{ω}-nitro-L-arginine, phenylmethylsulphonylfluoride, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and all antibodies used for immunohistochemistry were from Sigma (Deisenhofen, Germany), L-glutamine and penicillin/streptomycin solution were purchased from Serva (Heidelberg, Germany) and Gibco (Eggenstein, Germany), respectively. Gentamicin and trypsine/EDTA solution were obtained from Boehringer Mannheim (Mannheim, Germany). Foetal calf serum was from eurobio (Raunheim, Germany) and Boehringer Mannheim (Mannheim, Germany). (±)-Isoprenaline HCl was from Boehringer Ingelheim (Ingelheim, Germany). Tissue culture flasks from Becton Dickinson (Heidelberg, Germany) and Sarstedt (Nümbrecht, Germany) were used for the experiments without coating. All other chemicals used were of analytical or best commercial grade available.

Statistics

Results are expressed as mean ± s.e.mean. Significance was estimated by analysis of variance for repeated measures, two-way analysis of variance with least square differences range test or Student's *t* test for unpaired observations as appropriate. pEC_{50} and pIC_{50} values are defined as $-\log$ mol l^{-1} of EC_{50} and IC_{50} values, respectively. A P value less than 0.05 was considered significant.

Results

Influence of cantharidin on force of contraction in bovine isolated coronary artery rings (CARs)

Cantharidin (100 μM) increased force of contraction in CARs with and without endothelium in a time-dependent manner. A significant increase in force of contraction compared to basal tension was detected after 30 min of exposure. Maximal effects (322 ± 24 mN for CARs with endothelium and 279 ± 44 mN for CARs without endothelium) were observed after 75 min (Figure 1a). Cantharidin at lower concentrations (30 μM and 10 μM) also increased force of contraction in CARs with endothelium, but the maximum effects observed were smaller (200 ± 26 mN and 201 ± 63 mN) and occurred later, namely after 120 and 165 min, respectively. In CARs without endothelium the maximum effects to cantharidin were 170 ± 19 mN for 30 μM and 153 ± 33 mN for 10 μM and were observed after 135 and 180 min, respectively. Cantharidin at concentrations from 0.1 – 3 μM did not increase force of contraction in CARs with and without endothelium within 180 min (Figure 1b). Control rings treated with an appropriate amount of solvent (DMSO) showed no significant changes in force of contraction within 180 min compared to basal (resting) tension. Our data demonstrate that cantharidin increased force of contraction in bovine isolated coronary artery rings in a time- and concentration-dependent manner. The vasoconstrictor effects of cantharidin are endothelium-independent with similar pEC_{50} values of about 5.1 and 5.2, for both preparations. Therefore, all experiments in isolated rings focussing on possible effects of cantharidin on vasorelaxation were performed with 3 μM cantharidin because this was the highest concentration of cantharidin devoid of vasoconstrictor effects.

Effects of cantharidin on isoprenaline- or 3-isobutyl-1-methylxanthine-induced vasorelaxation in bovine isolated coronary artery rings (CARs)

Bovine coronary artery rings were incubated for one hour with cantharidin (3 μM) or DMSO (solvent control), respectively, and contracted with KCl (75 mM). Cantharidin (3 μM) alone did not affect force of contraction in endothelium intact rings compared to control rings. Isoprenaline (100 μM)-induced vasorelaxation was attenuated by 3 μM cantharidin in a time-dependent manner from 45 ± 6 to 98 ± 13% of KCl value after 60 min in CARs with endothelium (Figure 2b). and from 40 ± 4 to 82 ± 12% of KCl value after 60 min in CARs without endothelium (Figure 2c). Cantharidin (3 μM) attenuated isoprenaline (0.03 – 100 μM)-induced vasorelaxation in CARs with (Figure 3a, 30 min and Figure 3b, 60 min) and without endothelium (data not shown). In contrast, 3 μM cantharidin did not attenuate the 3-isobutyl-1-methylxanthine (10 – 100 μM)-induced vasorelaxation in CARs with (Figure 4a, IBMX 100 μM) and without endothelium (Figure 4b, IBMX 100 μM).

Because in these experiments the effects of cantharidin (3 μM) were assessed against different levels of relaxation, additional experiments were performed in rings where similar levels of relaxation occurred, namely 59 ± 6% of KCl value for isoprenaline (1 μM, Figure 5a) and 65 ± 5% of KCl value for IBMX (30 μM, Figure 5b). Also, in these experiments 3 μM cantharidin attenuated vasorelaxation induced by isoprenaline,

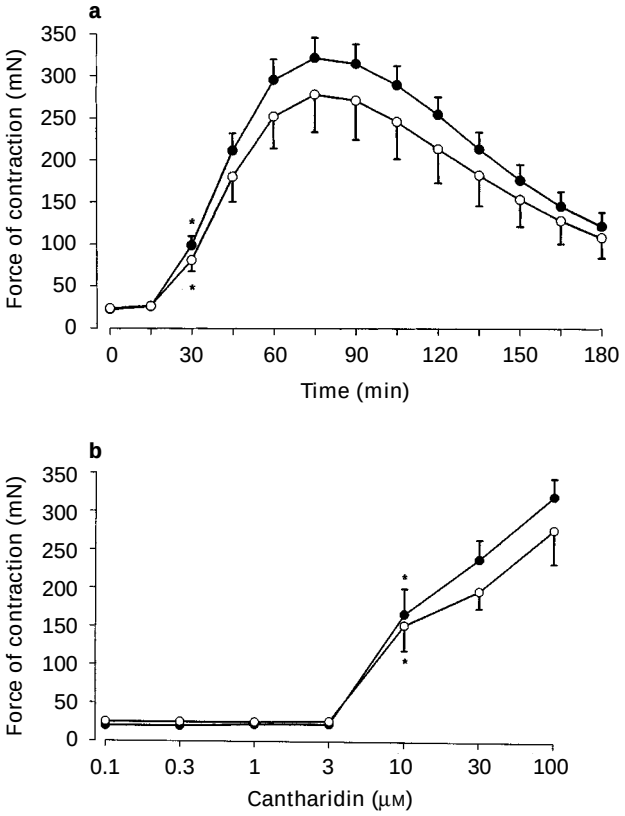

Figure 1 Influence of cantharidin on force of contraction in bovine isolated coronary artery rings (CARs). (a) Bovine isolated coronary artery rings were exposed to cantharidin (100 μM) and force of contraction in rings with endothelium (●) and without endothelium (○) was measured at the times indicated. Control rings treated with an appropriate amount of solvent (DMSO) showed no significant changes in force of contraction within 180 min compared to basal tension (data not shown). Values represent mean and vertical lines s.e.mean of at least 8 experiments. No significant difference between CARs with and without endothelium were noted comparing both lines by analysis of variance (ANOVA for repeated measures). The asterisks denote the first significant increase in force of contraction compared to predrug value. (b) Bovine isolated coronary artery rings were exposed to cantharidin (0.1 – 100 μM) in a non-cumulative manner. The maximum force of contraction in CARs with endothelium (●) and without endothelium (○) within 180 min was plotted. Control rings treated with an appropriate amount of solvent showed no significant changes in force of contraction within 180 min compared to basal tension (data not shown). Values represent mean and vertical lines s.e.mean of the maximum effects of at least 4 experiments. No significant differences between CARs with and without endothelium were noted comparing both lines by two-way analysis of variance with least square differences range test. The asterisks denote the first significant increase in force of contraction compared to predrug value.

whereas vasorelaxation induced by IBMX was not attenuated. Furthermore, the relaxing effect of isoprenaline was not attenuated by 1 μM cantharidin (data not shown).

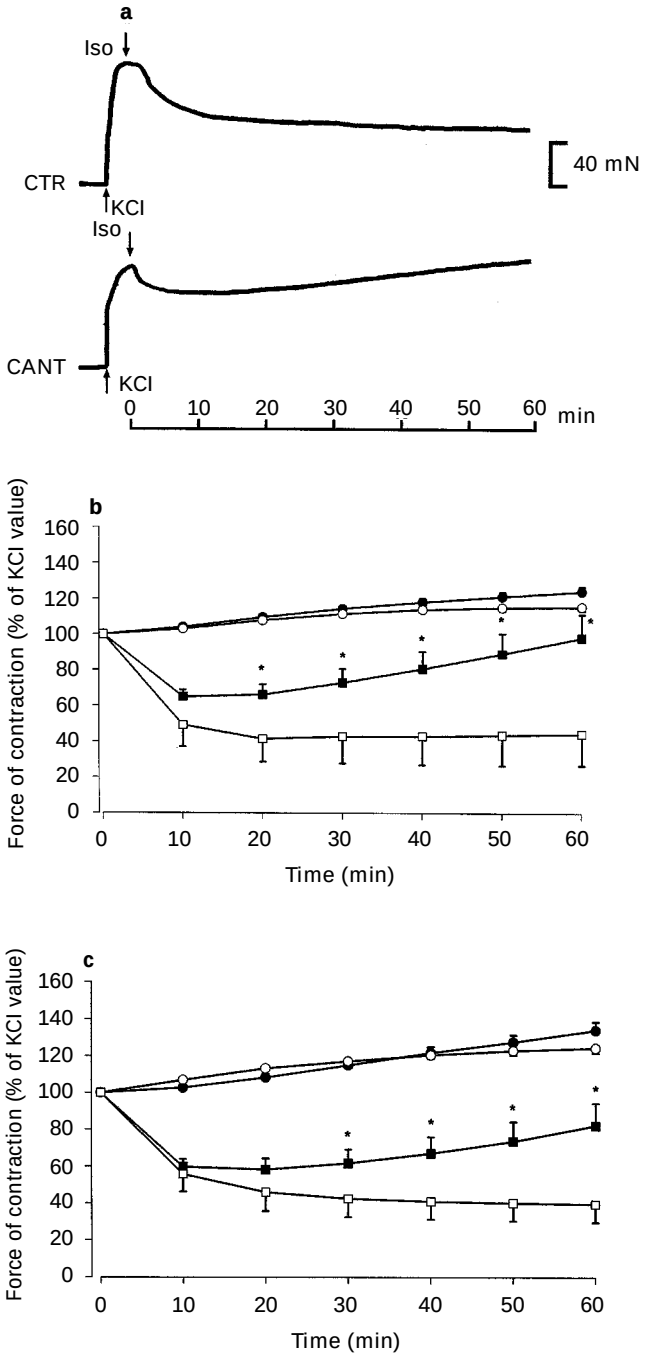

Figure 2 Influence of cantharidin (3 μM) on isoprenaline (100 μM)-induced vasorelaxation in bovine isolated coronary artery rings (CARs). (a) Two original recordings are depicted. CANT represents one intact ring treated with cantharidin (3 μM) and then isoprenaline (Iso, 100 μM), CTR represents one intact ring treated with an appropriate amount of solvent and then isoprenaline (100 μM). CARs with endothelium (b) and without endothelium (c) were precontracted with KCl (75 mM) and exposed to isoprenaline (100 μM) in the presence of solvent (□) or 3 μM cantharidin (■), respectively. Control rings in the presence of solvent (○) and 3 μM cantharidin (●) alone are plotted. Force of contraction at the times indicated is expressed as % of tone induced by predrug (KCl 75 mM). Values represent mean and vertical lines s.e.mean of at least 4 experiments. Cantharidin (3 μM) significantly attenuated vasorelaxation of CARs induced by isoprenaline compared to CARs treated with solvent and isoprenaline. Significance was estimated by comparing the lines by analysis of variance (ANOVA for repeated measures). The asterisks denote significant differences versus CARs treated with solvent and isoprenaline.

Influence of cantharidin (3 μM) on cyclic AMP content in bovine cultured vascular cells after β-adrenoceptor stimulation (Iso) or inhibition of phosphodiesterase activity (IBMX)

The effects of cantharidin on cyclic AMP content were investigated in bovine cultured vascular cells. We chose 3 μM cantharidin for these experiments on distinct but functionally relevant cell types because of reasons of comparability with our physiological data. Cantharidin 3 μM alone did not affect cyclic AMP content in bovine coronary artery smooth muscle cells (BCs, Figures 6a and 7a), bovine aortic endothelial cells (BAECs, Figures 6b and 7b) and bovine aortic smooth muscle cells (BASMCs, Figures 6c and 7c). Moreover, 3 μM cantharidin also failed to affect cyclic AMP content in BCs after β-adrenoceptor stimulation (Iso) or phosphodiesterase inhibition (IBMX) (Figures 6a and 7a). Similar results were obtained in aortic endothelial cells (BAECs, Figures 6b and 7b) and aortic smooth muscle cells (BASMCs, Figures 6c and 7c).

Protein phosphatase activity in homogenates of bovine coronary arteries and vascular cells

In homogenates of bovine coronary arteries, cantharidin inhibited phosphatase activity with a pIC_{50} of about 6.0 (Figure 8a). pIC_{50} values of cantharidin amounted to 6.5 in homogenates of bovine coronary artery smooth muscle cells (Figure 8b), 6.7 in homogenates of bovine aortic endothelial cells (Figure 8c) and 6.7 in homogenates of bovine aortic smooth muscle cells (Figure 8d).

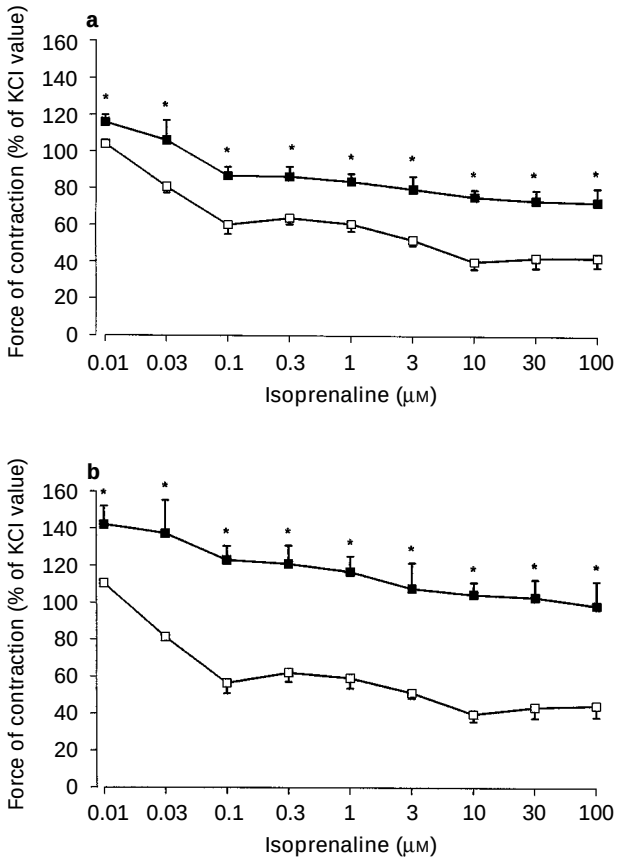

Figure 3 Influence of cantharidin (3 μM) on isoprenaline (0.01–100 μM)-induced vasorelaxation in bovine isolated coronary artery rings (Cars). Cars with endothelium were precontracted with KCl (75 mM) and exposed to isoprenaline (0.01–100 μM) in the presence of solvent (□) or 3 μM cantharidin (■), respectively. Force of contraction after 30 min (a) and 60 min (b) is expressed as % of tone induced by KCl (75 mM). Values represent mean and vertical lines s.e.mean of at least 3 experiments. The asterisks denote significant differences versus rings treated with solvent and isoprenaline.

Discussion

Cantharidin, an inhibitor of protein phosphatase types 1 and 2A, increased force of contraction in bovine isolated coronary artery rings with pEC_{50} values of 5.1 and 5.2 for CARs with and without endothelium, respectively (Figure 1a and b). In contrast, okadaic acid caused both vasodilatation and vasoconstriction in different experimental systems. Long-lasting contraction of human umbilical arteries, rabbit aorta and guinea-pig taenia caecum due to treatment with okadaic acid were observed by Shibata *et al.* (1982), whereas okadaic acid also caused endothelium-independent vasodilatation in pig coronary artery and dog basilar artery precontracted with K^+ (40 mM; Ashizawa *et al.*, 1989). Moreover, vasodilatation of canine basilar arteries even under resting tension was demonstrated by Kimura *et al.* (1993). The latter findings are in contrast to our results in bovine coronary arteries where cantharidin only elicited vasoconstriction under resting conditions. In additional experiments with calyculin A, another protein phosphatase inhibitor, calyculin A (0.3 μM) increased force of contraction in CARs from 24 ± 2 mN to 30 ± 2 mN after 30 min ($n = 4$), whereas 0.1 μM calyculin A failed to affect force of contraction. Experiments with higher concentrations of calyculin A were not performed because of the prohibitive costs of calyculin A needed in these organ bath experiments. Cantharidin and calyculin A are structurally not related but are both known to inhibit protein phosphatase activity. Both compounds led to vasoconstriction of bovine isolated coronary artery rings. These findings strengthen our hypothesis that the vasoconstrictor effects of cantharidin might be due to inhibition of protein phosphatases. Nevertheless, we cannot completely rule out possible membrane or intracellular effects of cantharidin other than phosphatase inhibiton.

Vascular smooth muscle relaxation is mediated at least in part by cyclic AMP-induced activation of cyclic AMP-dependent protein kinase with subsequent phosphorylation of various protein substrates like, for example, myosin light chain kinase and phospholamban, ultimately resulting in the dephosphorylation of myosin light chains and reduction of intracellular Ca^{2+} (Lincoln *et al.*, 1990; Lincoln & Cornwell,

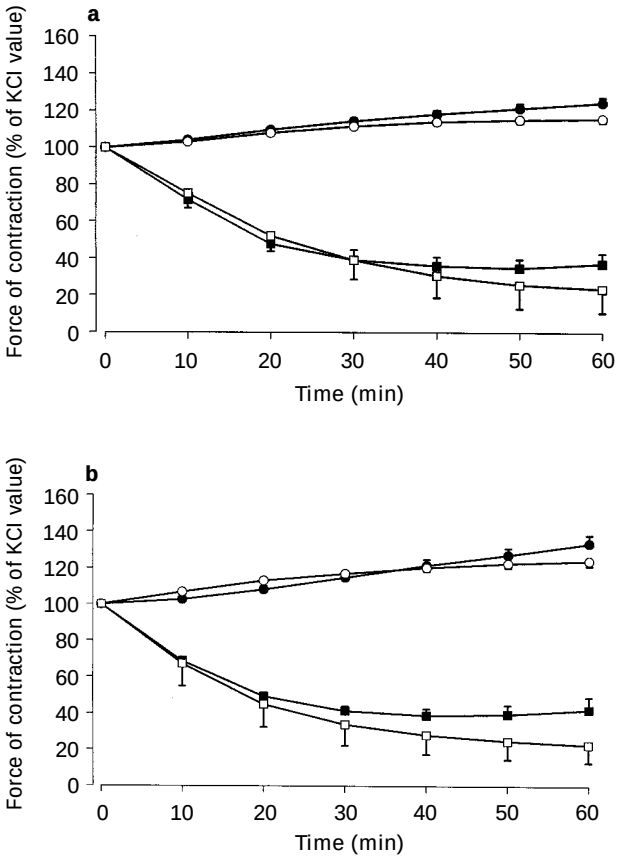

Figure 4 Influence of cantharidin (3 μM) on 3-isobutyl-1-methylxanthine-induced vasorelaxation in bovine isolated coronary artery rings (CARs). CARs with endothelium (a) and without endothelium (b) were precontracted with KCl (75 mM) and exposed to 3-isobutyl-1-methylxanthine (100 μM) in the presence of solvent (DMSO, □) or 3 μM cantharidin (■), respectively. The controls in the presence of solvent (○) and 3 μM cantharidin (●) alone are plotted. Force of contraction at the times indicated is expressed as % of tone induced by KCl (75 mM). Values represent mean and vertical lines s.e.mean of at least 5 experiments. No significant differences were noted between rings treated with cantharidin (3 μM) and 3-isobutyl-1-methylxanthine and rings treated with solvent and 3-isobutyl-1-methylxanthine by comparing the lines by analysis of variance (ANOVA for repeated measures).

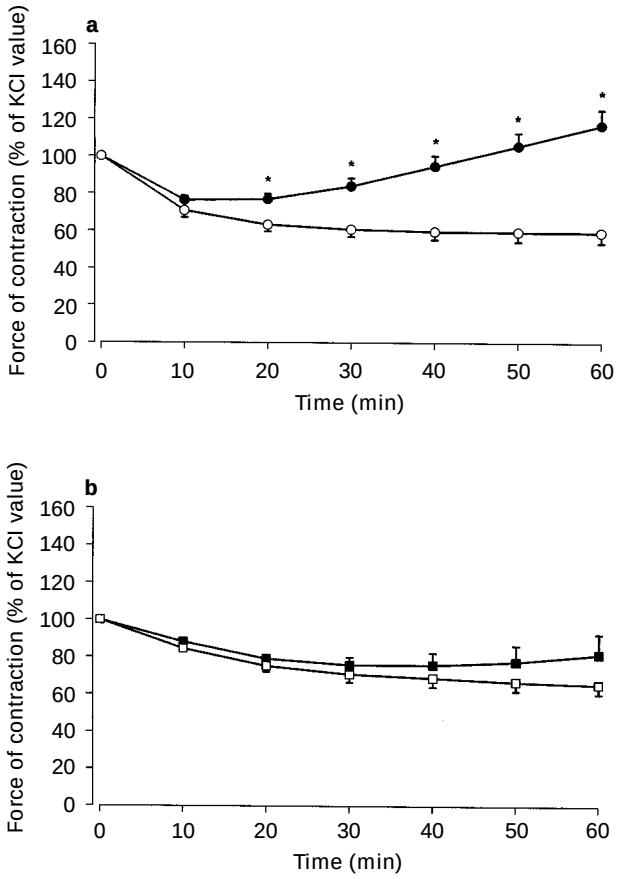

Figure 5 Influence of cantharidin (3 μM) on similar levels of relaxation in bovine isolated coronary artery rings (CARs). (a) CARs with endothelium were precontracted with KCl (75 mM) and exposed to 1 μM isoprenaline in the presence of solvent (○) or 3 μM cantharidin (●), respectively. Force of contraction at the times indicated is expressed as % of tone induced by KCl (75 mM). Values represent mean and vertical lines s.e.mean of at least 4 experiments. Cantharidin (3 μM) significantly attenuated vasorelaxation of CARs induced by isoprenaline compared to CARs treated with solvent and isoprenaline. Significance was estimated by comparing the lines by analysis of variance (ANOVA for repeated measures). The asterisks denote significant differences versus CARs treated with solvent and isoprenaline. (b) CARs with endothelium were precontracted with KCl (75 mM) and exposed to 30 μM 3-isobutyl-1-methylxanthine in the presence of solvent (□) or 3 μM cantharidin (■), respectively. Force of contraction at the times indicated is expressed as % of tone induced by KCl (75 mM). Values represent mean and vertical lines s.e.mean of at least 4 experiments. No significant differences were noted between rings treated with cantharidin (3 μM) and 3-isobutyl-1-methylxanthine and rings treated with solvent and 3-isobutyl-1-methylxanthine by comparing the lines by analysis of variance (ANOVA for repeated measures).

1993). Therefore, vascular smooth muscle contraction results at least in part from inhibition of protein phosphatases activity leading to enhanced phosphorylation of e.g. myosin light chains (Ozaki *et al*., 1987b; Allessi *et al*., 1992). To investigate the interaction between protein phosphatase inhibition and vasorelaxation due to elevation of cyclic AMP content via β-adrenoceptor stimulation, the effects of cantharidin on isoprenaline-induced vasorelaxation were examined in bovine isolated coronary artery rings. Additional experiments were performed with 3-isobutyl-1-methylxanthine, known to elevate cyclic AMP content via the inhibition of activity of phosphodiesterases. Bovine isolated coronary artery rings with or without endothelium, respectively, showed no significant difference in isoprenaline-induced vasorelaxation indicating that the relaxing response due to β-adrenoceptor stimulation is not dependent on intact endothelium. Moreover, treatment of intact isolated rings with the NO-synthase inhibitor N^{ω}-nitro-L-arginine (100 μM) for 15 min did not alter either basal tone, K^+ induced contraction or the relaxing effect of isoprenaline ($n=4$; data not shown). Thus, involvement of the NO pathway

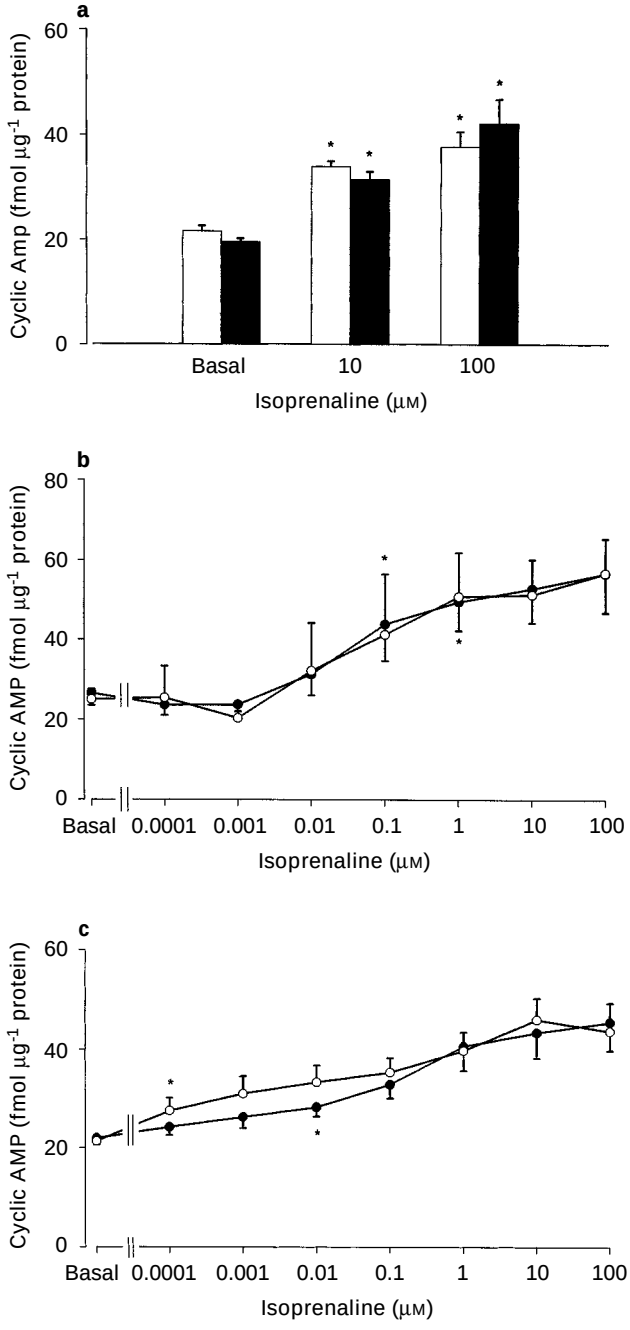

Figure 6 Influence of cantharidin (3 μM) on cyclic AMP content in cultured bovine vascular cells after β-adrenoceptor stimulation. Bovine coronary artery smooth muscle cells (BCs, a), aortic endothelial cells (BAECs, b) and aortic smooth muscle cells (BASMCs, c) were stimulated with isoprenaline for 30 min in the presence of cantharidin (3 μM) or solvent (DMSO), respectively. Open symbols (columns) represent cells treated with solvent, solid symbols (columns) represent cells pretreated with 3 μM cantharidin. Values represent mean and vertical lines s.e.mean of at least 4 experiments. The asterisks denote significant difference (a) or the first significant difference (b,c) versus basal values.

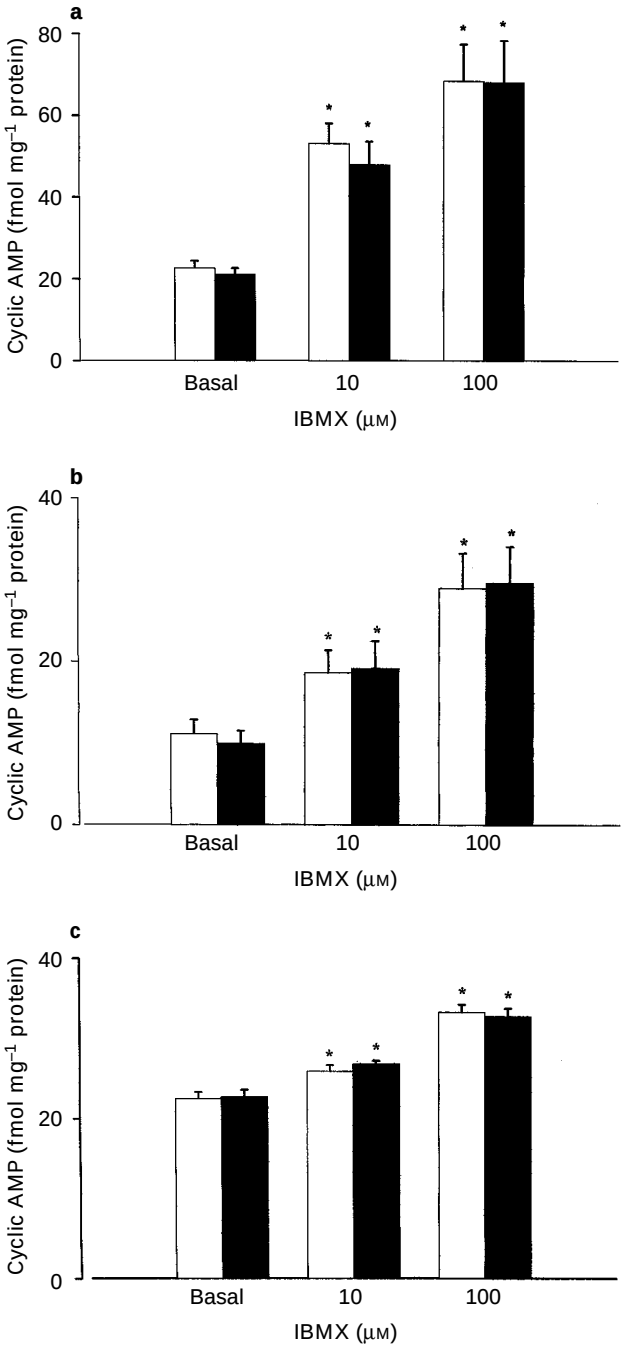

Figure 7 Influence of cantharidin (3 μM) on cyclic AMP content in bovine cultured vascular cells after inhibition of phosphodiesterase activity. (a) Bovine coronary artery smooth muscle cells (BCs), (b) bovine aortic endothelial cells (BAECs) and (c) bovine aortic smooth muscle cells (BASMCs) were stimulated with 3-isobutyl-1-methylxanthine (IBMX) for 30 min in the presence of 3 μM cantharidin (solid columns) or solvent (open columns), respectively. Values represent mean $\pm$ s.e.mean of at least 8 experiments. The asterisks denote significant difference versus basal values.

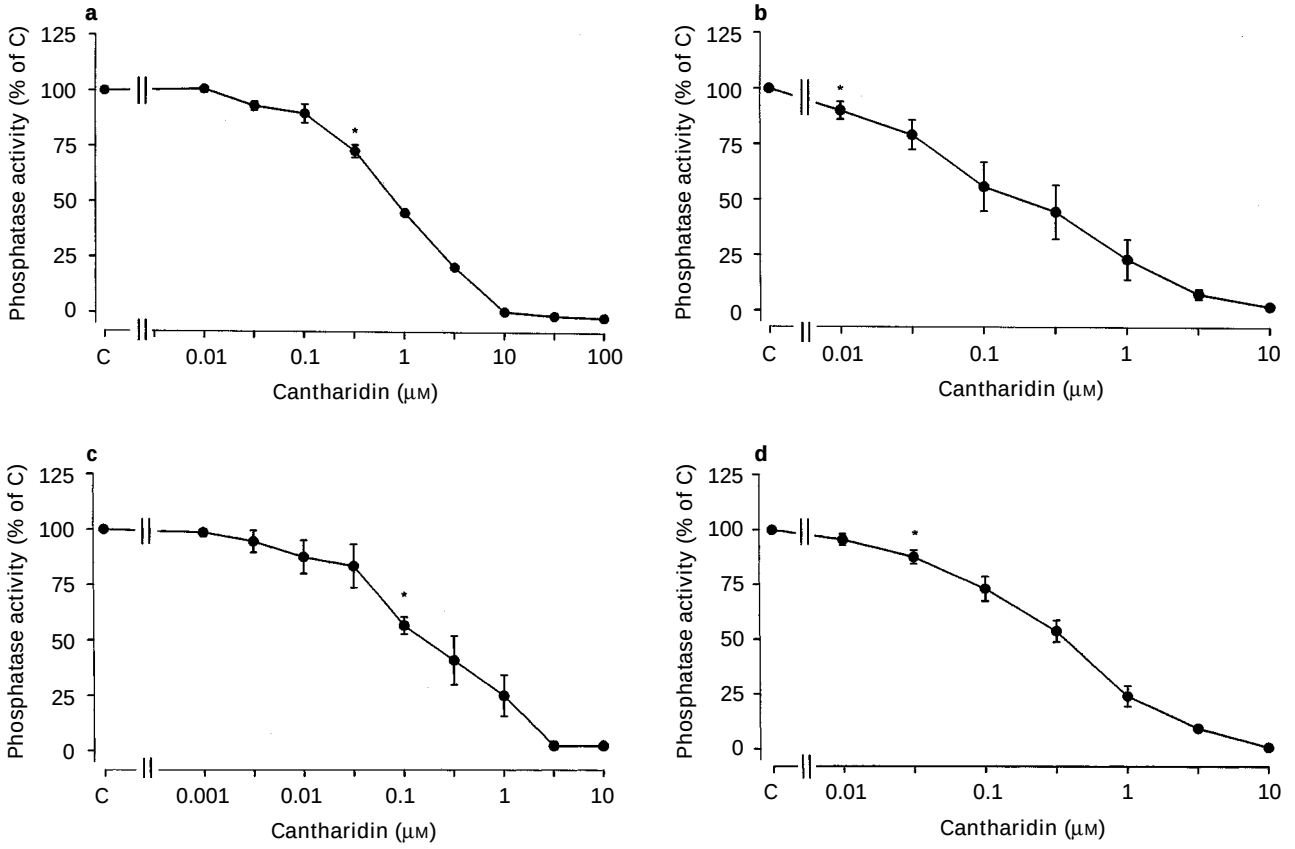

Figure 8 Protein phosphatase activity in homogenates of bovine coronary arteries and homogenates of cultured bovine vascular cells. Cantharidin inhibited phosphorylase a phosphatase activity in homogenates of bovine (a) coronary arteries, (b) coronary artery smooth muscle cells, (c) aortic endothelial cells and (d) aortic smooth muscle cells. Phosphorylase a phosphatase activity is expressed as % of solvent control (c). The concentration of solvent was constant under all experimental conditions. Values represent mean and vertical lines s.e.mean of at least 3 experiments. The asterisks denote the first significant difference versus control value.

in isoprenaline-induced vasorelaxation is very unlikely. These findings are in contrast to those in the rat thoracic aorta (Gray & Marshall, 1992), where relaxation due to isoprenaline treatment was found to be strictly dependent on intact endothelium. Whereas isoprenaline was able to relax isolated rings at concentrations ranging from 0.03 to 100 μM, vasorelaxation due to nonspecific inhibition of phosphodiesterase activity could only be observed at high concentrations of 3-isobutyl-1-methylxanthine (10–1000 μM) in CARs with and without endothelium.

Exposure of CARs with and without endothelium to 3 μM cantharidin only resulted in attenuation of vasorelaxation due to β-adrenoceptor stimulation by isoprenaline, whereas vasorelaxation due to inhibition of phosphodiesterase activity by IBMX remained unchanged. Because both compounds exert their biological effects at least in part by elevation of intracellular cyclic AMP levels, one could speculate that the attenuating effects of cantharidin on vasorelaxation after β-adrenoceptor stimulation could be due to decreased cyclic AMP levels due to antagonism by cantharidin of β-adrenoceptor agonist effects. Therefore, we investigated the influence of cantharidin on cyclic AMP accumulation in bovine cultured vascular cells. No alterations of cyclic AMP content could be observed either under basal conditions, after β-adrenoceptor stimulation with isoprenaline or inhibition of phosphodiesterase activity by IBMX, respectively. Therefore, it seems unlikely that cantharidin attenuates isoprenaline-induced vasorelaxation by antagonizing β-adrenoceptor agonist effects. Moreover, no evidence is available in the published literature. The crucial role of cyclic AMP in mediating vasorelaxation is supported by our findings that the greater maximum relaxation caused by IBMX than by Iso was accompanied by a greater maximum increase of cyclic AMP accumulation in bovine isolated coronary artery smooth muscle cells.

Cantharidin has been demonstrated by others to inhibit protein phosphatase activity with pIC_{50} values varying with the type of phosphatase and its substrate more than with the purity (Eldridge & Casida, 1995). Thus, pIC_{50} values were 7.3 for the native dimer form of PP2A from mouse liver cytosol assayed as phosphorylase a phosphatase activity (Li & Casida, 1992), 7.4 for PP2A and 6.3 for PP1 from rabbit skeletal muscle (Li *et al.*, 1993) and 6.8 for PP2A and 5.8 for PP1 in assays with phosphohistone as substrate (Honkanen, 1993). Moreover, cantharidin inhibited PP2A- and PP1-activity purified from guinea-pig ventricles with pIC_{50} values of 6.9 and 5.6, respectively, and was assumed to be a useful lead compound in the search for new positive inotropic agents (Neumann *et al.*, 1995). As depicted in Figure 8a–d, cantharidin inhibited protein phosphatase activity in homogenates of bovine coronary arteries and homogenates of bovine cultured vascular cells with pIC_{50} values ranging from 6 to 6.7. The difference between pIC_{50} values for phosphatase activity and the pEC_{50} values of cantharidin with respect to vasoconstriction might be due to differences in permeability between intact multicellular preparations (CARs) and homogenates of CARs and homogenates of bovine cultured vascular cells. Furthermore, the absence of protein kinase activity in the phosphatase assay system may contribute to these differences.

In summary, we demonstrate for the first time the inhibitory effects of cantharidin on protein phosphatase activity in vascular cells. Treatment of bovine isolated coronary artery rings with cantharidin resulted in a time- and concentration-dependent vasoconstriction which was endothelium-independent. As cantharidin inhibited protein phosphatase activity in homogenates of bovine coronary arteries and vascular cells, it is suggested that vasoconstriction due to cantharidin is at least in part mediated by phosphatase inhibition. Initial experiments indicate that cantharidin increases the phosphorylation state of

contractile proteins, e.g. myosin light chains, in smooth muscle cells (unpublished observations). The different effects of cantharidin on vasorelaxation due to β-adrenoceptor stimulation or inhibition of phosphodiesterase activity, cannot be explained by alterations of whole cell AMP content but may be due to compartmentation of cyclic AMP in the smooth muscle (Buxton & Brunton, 1983; Lobaugh & Blackshear, 1990). Because of the prohibitive costs of okadaic acid and calyculin A, cantharidin might be an economical tool to investigate the effects of protein phosphatase inhibition in the vascular system.

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